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PRINCIPAL INVESTIGATOR: Athar Chishti, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Chicago, IL 60612-7205

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14. ABSTRACT Neurofibromatosis type 2 is an autosomal dominant disorder, characterized by the development of bilateral vestibular and spinal schwannomas, meningiomas, and ependymomas. The NF2 gene encodes a 595 amino acid polypeptide known as NF2 protein or Merlin or Schwannomin. During the purification of NF2 protein from human erythrocytes, we noticed striking biochemical similarities between NF2 protein and p55. We have previously established that p55, a palmitoylated membrane phosphoprotein, forms a ternary complex with protein 4.1 and glycophorin C. Notably, the Drosophila homologue of p55 functions as a tumor suppressor in epithelial and neuronal tissues. To test the hypothesis that p55 is a major binding partner for NF2 protein in the RBC membrane, we generated maltose binding protein fusions of recombinant NF2 protein and tested their binding to p55. Both amino and carboxyl halves of NF2 protein were tested in the binding assay. Our results indicate a direct interaction between the FERM domain of NF2 protein and p55. The binding between p55 and NF2 protein was quantified, and existence of this complex was demonstrated in human erythrocyte plasma membrane. This unexpected finding reveals a new paradigm integrating the known functions of p55 family of proteins with the pathophysiology of NF2 protein.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Objective.....	4
Summary of the Work Completed.....	4
Experimental Procedures and Results.....	4
Conclusion.....	11
Training Accomplishments.....	11

Progress Report

Career Development Award # NF020087

PI: Athar H. Chishti

Title: Biochemical characterization of native Schwannomin.Merlin

OBJECTIVE

As outlined in the Statement of Work (SOW), Task # 2 proposed biochemical characterization of NF2 protein. Another objective was to initiate a screen for the binding partners of NF2 protein in the erythrocyte plasma membrane. In the last 6 months, we have made an important discovery by identifying a new binding partner of NF2 with functional implications in cell polarity and intracellular trafficking pathways.

SUMMARY OF THE WORK COMPLETED

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder, characterized by the development of bilateral vestibular and spinal schwannomas, meningiomas, and ependymomas. The NF2 gene encodes a 595 amino acid polypeptide known as NF2 protein or Merlin or Schwannomin. The primary structure of NF2 protein is homologous to the ERM family of peripheral membrane proteins, which includes Ezrin, Radixin, and Moesin. The founding member of the ERM superfamily is the erythrocyte membrane protein 4.1, which cross-link's spectrin-actin complexes and attaches them to the plasma membrane. During the purification of NF2 protein from human erythrocyte plasma membrane (ghosts), we noticed striking biochemical similarities between NF2 protein and p55. We have previously established that p55, a palmitoylated peripheral membrane phosphoprotein, forms a ternary complex with protein 4.1 and glycophorin C. Notably, the *Drosophila* homologue of p55 functions as a tumor suppressor in epithelial and neuronal tissues. To test the hypothesis that p55 is a major binding partner for NF2 protein in the RBC membrane, we generated maltose binding protein (MBP) fusions of recombinant NF2 protein and tested their binding to p55. Both amino and carboxyl halves of NF2 protein were tested in the binding assay. Our results indicate a direct interaction between the FERM domain of NF2 protein and p55. The binding between p55 and NF2 protein was quantified, and existence of this complex was demonstrated in human erythrocyte plasma membrane. This unexpected finding reveals a new paradigm integrating the known functions of p55 family of proteins with the pathophysiology of NF2 protein.

EXPERIMENTAL PROCEDURES AND RESULTS

Production of Recombinant NF2: NF2 N-terminus (aa 1-311) and C-terminus (aa 312-595) halves were amplified by the polymerase chain reaction (PCR) using human fetal brain cDNA pool (CLONTECH). The cDNA fragments were cloned into pMAL-c2X vectors (New England BioLabs), and the recombinant proteins were expressed in *Escherichia coli* strain, DH 5 α .

Production of Recombinant Protein 4.1: The FERM domain of human protein 4.1R, which starts from the second ATG (nucleotide 801) in exon 4 to phenylalanine in exon 12 (nucleotide 1694), was PCR-amplified from the protein 4.1 cDNA and cloned into pMAL-c2X vector (New England BioLabs). The recombinant protein was expressed in *Escherichia coli* strain, DH 5 α .

Production of Recombinant p55: cDNA of full-length p55 of human erythroid was obtained by PCR. The PCR-amplified product was cloned into pQE9 vector (QIAGEN), and the recombinant protein was expressed in *Escherichia coli* strain, DH 5 α . In addition, the cDNA was also cloned into pFastBac expression vector (GIBCO-BRL). The recombinant baculoviruses were generated by using the Bac-to-Bac baculovirus expression system (GIBCO-BRL) according to the manufacturer's protocol. Recombinant baculoviruses were amplified twice prior to infection of Sf9 cells, and two clones for each recombinant baculovirus stock were confirmed for recombinant full-length p55 DNA sequence by PCR with the same primers used for the cloning procedure. For each construct, four recombinants were amplified.

Site-directed Mutagenesis and expression of p55: The point mutation on amino acid 94 (cys to ala) in full-length p55 construct was created by site-directed mutagenesis. The primers used were 5'-TGAAAAACAGTCCGCTACGGTGGCCAGAATT-3' (sense) and 5'-AATTCTGGCCACCGTAGCGGACTGTTTTCA-3' (antisense). The product was cloned into pFastBac vector. Also, mutant baculoviruses were generated using the Bac-to-Bac baculovirus expression system.

Bacterial expression of NF2 N- and C-terminus halves and full-length p55: A single bacterial colony containing the desired plasmid was grown overnight at 37 °C and diluted in 300 ml of culture media, and grown to mid-log phase before induction with 0.2 mM IPTG. The cell pellet was resuspended in 20 ml of lysis buffer containing 8.0 mM sodium phosphate, pH 7.3, 1.0 mM EDTA, 1% Triton X-100, and 72 mM 2-mercaptoethanol, and was lysed by brief sonication. The supernatants of NF2 N- and C-terminus halves were purified on amylose resin. Beads bearing fusion proteins were stored on ice. In addition, the supernatant of full-length p55 was purified on Ni⁺⁺ column (INVITROGEN).

Expression of wild-type and mutant recombinant p55s in insect cells: Expression of recombinant p55 was evaluated in cell lysates and the medium of Sf9 cultures. The Sf9 cells, grown in monolayers, were infected at a multiplicity of infection (MOI) of 10 with the appropriate recombinant baculovirus, and the cells were collected 36 h postinfection (hpi). The expression of the recombinant p55 protein in the medium was analyzed by infection of a 200-ml insect cell culture at a density of 2×10^6 cells/ml (4×10^8 total cells per spinner flask) with an MOI of 5. Cells were harvested and recombinant p55 was purified from the cell lysate using Ni⁺⁺ column (INVITROGEN).

In Vitro NF2 N- and C-terminus halves Binding Assay with Full-length p55

Expressed in Bacteria and in Insect cells: NF2 N- and C-terminus halves were immobilized to amylose resin in TBS-Tween20 for 2 hours in 4°C. Lysate containing full length p53 was diluted 10-fold in TBS-T, and the respective MBP fusion proteins were added to each tube followed by incubation for 2 hours at 4°C. Binding of MBP fusion NF2 N- and C-terminus halves to full-length p53 were assessed by maltose binding protein (MBP) pull down assay. Beads were recovered by centrifugation, washed, and bound protein was analyzed by SDS-PAGE. The gel was stained by Coomassie Blue.

SDS-PAGE and Western blot: Proteins were resolved by SDS-PAGE (10% acrylamide) and electro-transferred onto the nitrocellulose membrane (Hybond-C). The nitrocellulose blot was blocked with the blocking buffer (5% milk; 0.1% Tween 20, 10 mM Tris-HCl, and 150mM NaCl in TBS, pH 7.5) and incubated with anti-p53 monoclonal antibodies at 1: 5000 dilution in the blocking buffer for 2 hours at room temperature. Following incubation, blots were washed with the TBS-T buffer (0.1% Tween 20, 10 mM Tris-HCl, and 150mM NaCl in TBS, pH 7.5), and incubated for 1 hour with goat anti-mouse IgG HRP at 1: 5000 in blocking buffer. After washing with the TBS-T, the p53 protein was visualized using the ECL Western blotting detection system.

Surface Plasmon Resonance Measurements: Protein interactions were quantified using BIAcore Optical Biosensor 1000 (Biacore Inc). To analyze the interaction between MBP-NF2 N and WTP53, MBP-NF2 C and wild type P53, MBP-NF2 N and MBP-NF2 C fusion protein were passed over the immobilized wild type P53 surface. The wild type p53 was used at a concentration of 200nM. In kinetic experiments, the concentration of MBP fusion proteins of NF2 N and NF2C ranged from 20 nM to 120 nM. The experiments were carried out at 25 °C at a flow rate of 30 µl/min for the kinetic measurements, except the immobilization and regeneration processes, which were carried out at a flow rate of 5.0 µl/min. The composition of the running buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20. The immobilization buffer for wild type P53 was at a pH of 3.5, 10 mM Sodium Acetate, and the regeneration buffer had 100 mM NaCl and 10 mM NaOH.

Our binding assays demonstrate that the N-terminal FERM domain of NF2 protein specifically interacts with the human erythrocyte p53, produced either in bacteria or in insect cells (Figs. 1-3). In contrast, the C-terminal half of the NF2 protein failed to associate with p53. Surface plasmon resonance measurements confirmed the findings made by the bead pull-down assay and quantified the affinity of the interaction in the 3-4 nM range (Figs. 4-5). Together, our results identify a new interaction of NF2 protein in the erythrocyte plasma membrane.

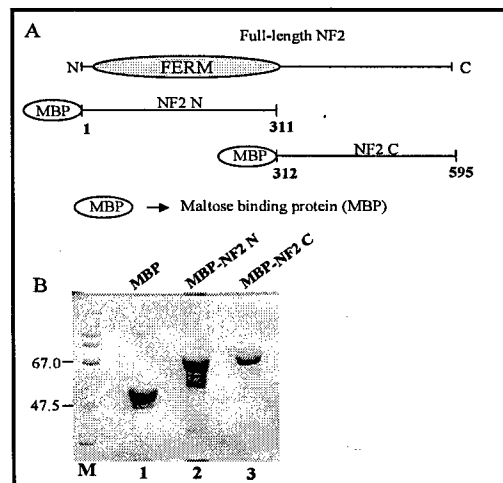


Fig. 1. Recombinant proteins of NF2. A, a schematic diagram of NF N- and C-terminal constructs made for the experiments. The NF2 N corresponds to N-terminal half (311 a.a) that includes whole FERM domain, and NF2 C is C-terminal half (283 a.a). B, bacterial expressed MBP fusion proteins expressed in bacteria were analyzed by SDS-PAGE.

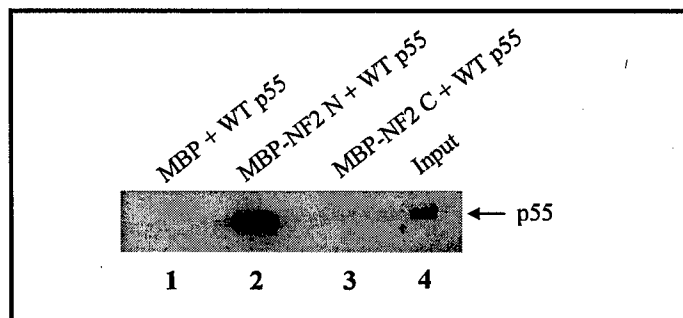


Fig. 2. Western blot of *in vitro* NF2 N- and C-terminus halves binding assay with full-length p55 expressed in bacteria. Full-length p55 was detected by anti-p55 monoclonal antibody.

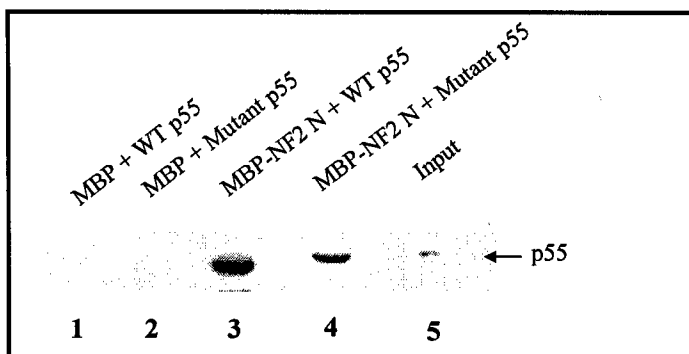


Fig. 3. Western blot of *In Vitro* NF2 N half binding assay with full-length p55 expressed in bacteria and in Insect cells.

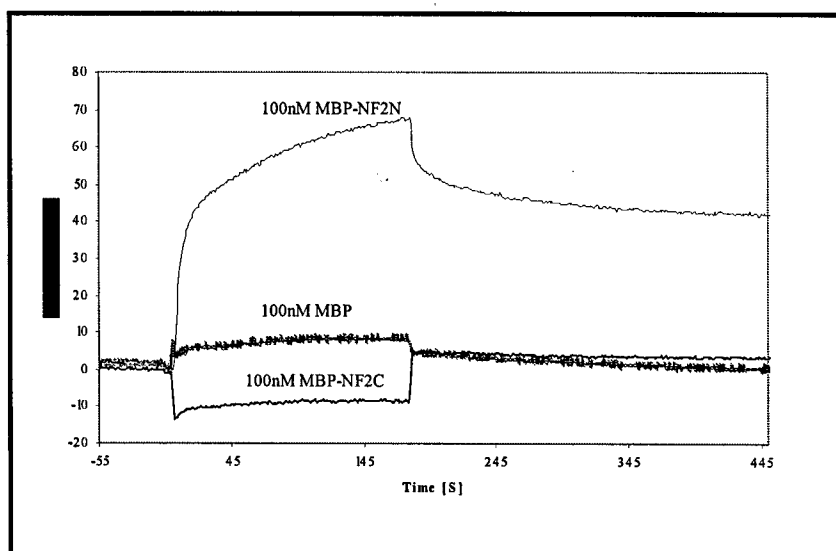


Fig. 4. Measurements of the interaction between wild type p55 and MBP-NF2 N fusion protein, wild type p55 and MBP-NF2 C fusion protein, and wild type p55 and MBP protein. Responses in RU are plotted as a function of time (sensorgrams). Wild type p55 were injected at a concentration of 200 nM over immobilized and a total of 280 RU of p55 was immobilized on the CM5 sensor chip (BIAcore Inc.). Binding was performed at a flow rate of 30 μ l/min, 25 $^{\circ}$ C. No interactions were observed in wild type p55 and MBP-NF2 C fusion protein, wild type p55 and MBP protein.

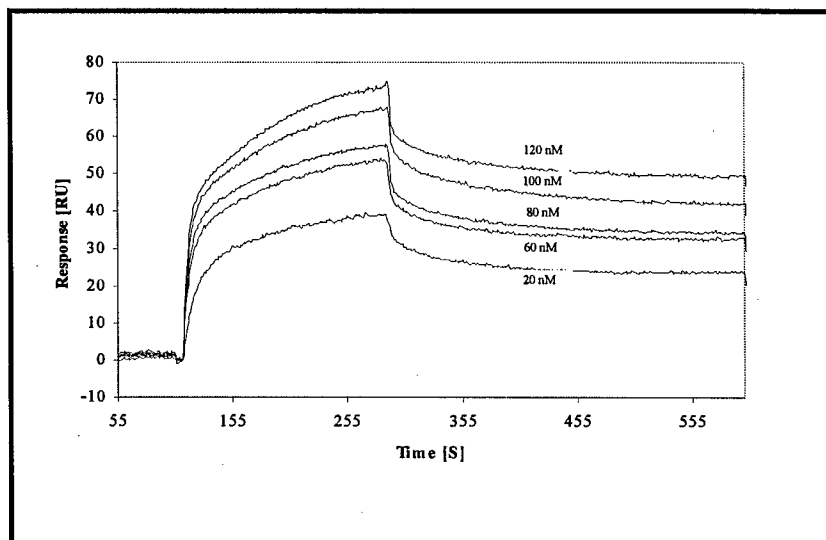


Fig.5. The interaction between MBP-NF2 N fusion protein with wild type p55 at different concentrations for the kinetic analysis. A total of 280 RU of p55 was immobilized on the CM5 sensor chip, and binding was carried out at a flow rate of 30 $\mu\text{l}/\text{min}$ at 25 $^{\circ}\text{C}$. The data were analyzed with BIAevaluation 3.0 software and a conformation change model was chosen because it is the most appropriate compared with other models.

The conformation change model is: $A+B \rightarrow AB \rightarrow AB^*$ (analyte (A) binds to ligand (B); complex AB changes to AB^* , which cannot dissociate directly to $A+B$). In the first step $A+B \rightarrow AB$, association rate constant (k_{a1}) obtained was $(86.9 \pm 6) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and dissociation rate constant (k_{d1}) obtained was $(55.8 \pm 5) \times 10^3 \text{ s}^{-1}$. In the second step $AB \rightarrow AB^*$, association rate constant (k_{a2}) obtained was $(122 \pm 7) \times 10^{-4} \text{ s}^{-1}$, and dissociation rate constant (k_{d2}) obtained was $(69.1 \pm 4) \times 10^5 \text{ s}^{-1}$. An equation: $(k_{a1}/k_{d1}) * (k_{a2}/k_{d2})$ was used to calculate the association equilibrium constant (K_A). The association equilibrium constant (K_A) obtained was $2.74 \times 10^8 \text{ M}^{-1}$, and the dissociation equilibrium constant (K_D) was 3.7 nM.

CONCLUSIONS

The MBP fusion proteins of N- and C- halves of NF2 protein were expressed in bacteria and purified in soluble form.

NF2 protein binds to erythrocyte membrane protein p55 by *in vitro* binding assay and BIAcore surface plasmon resonance measurements. The amino-terminal FERM domain of NF2 protein encodes the binding site for p55.

The affinity (K_D) of the interaction between NF2 N and recombinant p55 is 3.7 nM, as measured by the surface plasmon resonance assay.

Our previous results have shown that NF2 protein is present in the protein 4.1 null red blood cell membranes, which also lack p55, as well as glycophorin C. This observation suggests that the p55-NF2 protein interaction may be functionally more significant in the non-erythroid cells. Experiments are currently underway to examine the role of p55-NF2 protein interaction in Schwann cells.

TRAINING ACCOMPLISHMENTS

Financial support provided under the Career Development Award enabled Dr. Chishti to utilize his extensive experience of erythrocyte membrane biochemistry in discovering new biochemical properties of NF2 protein. This focus finally led to the discovery of p55 as the binding partner for NF2 protein. The p55 protein is a major focus of Dr. Chishti's research interests. All necessary reagents are in place, and the p55 knockout mouse phenotype is currently under investigation. These resources will be invaluable for the future characterization of p55-NF2 protein interactions by Dr. Chishti and other laboratories. To expand this program further, a new postdoctoral fellow, Dr. Pil Seo, has been recruited to work on the p55-NF2 protein interactions. Dr. Seo is currently investigating the functional significance of p55-NF2 protein interaction in non-erythroid cells.